Root Clearing/Fungal Staining in Tropical Trees and Vines

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Highly modified from this protocol:
https://plantscience.psu.edu/research/labs/roots/methods/methods-info/staining-of-mycorrhizal-fungi

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10% KOH - Slowly removes tannins and non-fungal structures, heat makes the reaction occur faster

- Remove ethanol from vials and rinse roots thoroughly, ensuring root surface is clean
- Add 10% KOH to vials as the basic step for all following protocols
- Roots can be removed from KOH, rinsed, and checked microscopically throughout this process to confirm correct clearing
- **Method #1: 1hr water bath at 95°C**
    - Place KOH filled vials in water bath for 1 hour at 95°C
    - This is what is in most original protocols, but I found my roots were still dark
    - At a certain point of tannin absorption by the KOH, the reaction stops so KOH must be replaced
    - It is always good to try this at first and reassess further steps after
    - Repeated heat baths were too intense for roots and caused degradation, so an altered protocol was needed
- **Method #2: 24hr room temp, replace KOH, 1hr water bath at 95°C**
    - Leave KOH until the next day
    - If KOH is dark, replace and add fresh KOH
    - Place in water bath for 1 hour at 95°C
    - Clearing initially at room temperature allowed better control over clearing roots
    - After 24hr white and light roots should be clear without the water bath
    - Roots that are brown or dark brown will need a water bath step after replacing the KOH
    - Most of my roots were still dark after both of these steps, so a further protocol was needed
- **Method #3: 48hr room temp, replace KOH, 1hr water bath at 95°C**
    - Same protocol at #2 but leave roots for two days
    - After 48hr the KOH was dark in most samples and replaced

    - After the water bath, more than half of the roots were still dark
    - Method #2 and #3 were used to reduce root exposure to heat longer than 1 hour, however, this was not effective and took too long
- **Method #4: 1hr water bath at 95°C, overnight room temp, replace KOH, repeat**
    - Because of the variation in roots and samples, an iterative protocol worked the best for me
    - Roots should be checked at each step and the protocol should be stopped when roots become light yellow to white
    - Initially heat the roots in KOH with the water bath to remove a bulk of the tannins
    - Replace KOH if dark, and warm in water bath for 15min
    - Leave at room temperature in warm KOH overnight
    - Replace KOH if dark and repeat
    - This leads to a simplified protocol that is repeated depending on the darkness of the roots
        - 1hr water bath at 95°C
        - Leave at room temp overnight
        - If still dark, replace KOH repeat

3% Peroxide - Clears pigments

- Compared to HCl, peroxide is more gentile
    - It only targets pigments, not structure
- This step is good to add for stubborn roots that are becoming fragile
- In 10min the dark exterior whitens, but does little to clear internal structures
- If KOH has not cleared the cortex and xylem/phloem, peroxide will not help
- HCl step must still be done for correct staining

HCl - Clears remaining pigment and structures from the outer layer of roots

- Pour out KOH or peroxide from vial, rinse 3x with DI, and cover roots with HCl solution
- **Method 1: 2% HCl for 1hr**
    - This is okay for any roots yellow or lighter
    - Relatively long
- **Method 2: 5% for 20min**
    - This gives the same results as 2% but faster
    - Works for darker roots
    - Is more abrasive for fragile roots
- **Method 3: 20% for 5 min**
    - This is for yellow or light tan roots
    - Too strong for already fragile roots

Trypan Blue - Deposits pigment into fungal structures and certain macromolecules

- Roots can be removed from trypan blue, rinsed, and checked microscopically throughout this process to confirm correct staining
- For all below methods, pour out HCl but **do not rinse** roots or vial out
- **Method #1: 0.03% Trypan Blue, 30min water bath 95°C**
    - Basic protocol that I find too intense for most correctly cleared roots
    - This led to extremely dark roots
    - This amount of heat with acidified glycerol also led to more degradation
- **Method #2: 0.015% Trypan Blue, 30min water bath 95°C**
    - This resulted in lighter roots, but the degradation issue was still present
    - Roots were still relatively dark
- **Method #3: 0.015% Trypan Blue, overnight room temp**
    - Some roots were a little dark after destaining
    - Longer process
    - No degradation due to room temp
- **Method #4: 0.015% Trypan Blue for 2hr at room temp**
    - This process worked perfectly
    - Roots uptake enough pigment to stain root fully
        - This is determined by the vascular bundle being stained
    - Intense detain not needed due to accurate stain penetration, so roots can be analyzed instantly
- **Method #5: 0.03% Trypan Blue for 1hr at room temp**
    - This process was even better
    - It was faster and created more contrast between fungi and root
    - It is better to oversaturate and destain than the potentially missing fungal structures

Subtractive Staining

- Adding trypan to roots in large amounts
- Over a long period of time or with heat
- Pigment needs to be destained from plant cells
- Fungal cells hold onto pigment tightly
- Fungal contrast only occurs after destaining with acidified glycerol
- This process takes longer due to removing excess trypan

Additive Staining

- Adding little amount of pigment until fungal structures are visible
- This involved leaving roots in trypan for less than 2hr at room temp
- This allows the easily stained fungal cells to uptake pigment and plant cells not

- This is a quicker process due to reduced trypan exposure and no destain
- There is less pigment to destain, because the root was not saturated

Acidified Glycerol

- Destain, replace
    - When using subtractive pigment staining
    - Excess pigment from plant cells seeps into acidified glycerol
- Storage for additive
    - Over time pigment still seeps into glycerol
    - Analyze within 6 months, I plan to within a month
    - The root will lose all trypan if left too long, but can be restained?

Finalized Method:

Clearing

1. Pour out EtOH and rinse 3x with DI water
2. Add 10% KOH to vial
3. Place in water bath (95°C) for 1 hour
4. Leave samples at room temperature in the same hot KOH overnight
    - Replace KOH if dark and place back in water bath for 15min to warm before leaving overnight
5. If roots are still dark repeat steps 3 and 4, replacing KOH before if necessary
6. Pour out KOH and rinse 3x with DI
7. Place the darkest root on a slide and examine microscopically for the level of clarity
    - The cortex should be a light yellow to gray
    - It should be easy to focus on cells near and across the cortex
    - Clear longer if needed
8. Add 3% hydrogen peroxide to your vial and leave for 1 hour
9. Pour out and rinse 3x with DI water
10. Add 5% HCl and leave for 10 minutes
11. Pour out HCl, and **do not rinse**

Staining

12. Add 0.03% trypan blue to the vial and leave at room temp for 1 hour
    - Fungal structures should be blue
    - Plant cells should be light blue to almost white
    - Cortex of root should have blue pigment
    - if not stained properly, leave for 10 more minutes
13. Place the root on a slide and examine microscopically for the level of stain
14. Pour out trypan blue and rinse 3x with DI
15. Add acidified glycerol to store roots

Note: While holding vial up to light roots should be a translucent blue (opaque blue indicates not cleared enough or over stained),

Allowing time for extra stain to seep out is always better than immediate analysis (probably upper limit where fungi loose pigment)